

A STUDY OF PROGRAMMED CELL DEATH IN COTTON (*Gossypium hirsutum*)

FIBER

A Thesis

by

MEGHAN C. ROCHE

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

August 2007

Major Subject: Plant Breeding

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Approved by:

Co-Chairs of Committee,	Z. Jeffrey Chen
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ABSTRACT

A Study of Programmed Cell Death in Cotton (*Gossypium hirsutum*) Fiber.

(August 2007)

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Cotton fiber has been postulated to undergo a process of programmed cell death (PCD) during the maturation phase of development. A parallel may exist between cotton fibers and xylem tracheary elements, which have periods of elongation, secondary cell wall deposition and death. Secondary wall formation and PCD are purported to be coupled events in tracheary elements. In this study, an attempt was made to observe the occurrence and timing of PCD in cotton fibers by TUNEL staining to detect DNA strand breaks, and also to monitor DNA content by PI staining.

The staining patterns produced by PI and TUNEL left room for interpretation. TUNEL-positive and PI-stained areas were observed, but failure to observe nuclei of conventional appearance in my cytological preparations at any time-point, along with possible nonspecific staining or autofluorescence of cell wall and intracellular components, made it difficult to draw firm conclusions of significance. Thus, additional analyses will be needed to prove or disprove current PCD theories. Nevertheless, the differences in TUNEL and PI signals across fiber development stages indicate that the observed fluorescence patterns are marking discrete developmental phases. The PI signal is dispersed throughout the cell during the elongation phase (5-15 DPA) and appears to condense during secondary cell wall synthesis (25- 40 DPA). TUNEL-positive signal may be observed as early as 25 DPA, but the signal is not widespread until 45 DPA. At 50 DPA and beyond, PI staining is reduced. Visually detectable DNA can be extracted from cotton fiber nuclei between 5 and 40 DPA, although a laddering pattern was not visible at any time-point. The results, although inconclusive, point to the possibility that PCD may be a process leading to maturation in the cotton fiber, succeeding completion of secondary cell wall synthesis.

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NOMENCLATURE

AGP	Arabinogalactan Proteins
BR	Brassinosteroids
DPA	Days Post Anthesis
EtBr	Ethidium Bromide
HR	Hypersensitive Response
MS	Murashige and Skoog Medium
PI	Propidium Iodide
PCD	Programmed Cell Death
ROS	Reactive Oxygen Species
TdT	Terminal deoxynucleotidyl Transferase
TUNEL	Terminal deoxynucleotidyl transferase mediated dUTP Nick End Labeling
UV	Ultraviolet

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INTRODUCTION

Cotton (*Gossypium hirsutum*) fibers are elongated, terminally differentiated, seed trichomes. Cotton fiber development occurs over four overlapping developmental phases: initiation, elongation, secondary wall deposition, and maturation. Many factors are coordinated during development, including reactive oxygen species, brassinosteroid, callose and calcium, and arabinogalactans. Fiber cells begin to expand at anthesis, the day of flowering, and elongation continues for approximately 21-26 days thereafter. Secondary cell wall synthesis begins at approximately 16 days post anthesis (DPA), peaks at about 24 DPA (Arpat et al., 2004), and concludes between 32 and 40 DPA (Meinert and Delmer, 1977; John and Crow, 1992; Maltby et al., 1979). A period of desiccation and maturation follows between 45 and 60 DPA (Ji et al., 2003; Kim and Triplett, 2001). Fibers have been proposed to die sometime after 40 DPA by a method of programmed cell death (PCD) (Potikha et al., 1999).

Programmed cell death is an active and genetically controlled form of cell death that occurs in several plant developmental processes, including tracheary element formation (Groover and Jones, 1999) and senescence (Pennell and Lamb, 1997), as well as in the hypersensitive response (HR) to pathogen infection (Swidzinski et al., 2002). Hallmark features of PCD include nuclear condensation, marginalization of heterochromatin to the nuclear envelope (membrane blebbing), cytoplasmic vacuolization and DNA fragmentation, leading to reduced staining of fluorescent DNA dyes (Broderson et al., 2002; Gold et al., 1993). Nuclear DNA degradation follows shortly after chromatin condensation as nucleases fragment DNA into segments of approximately 50 kb. Chromatin is further fragmented into oligonucleosomal-sized fragments in multiples of 180 base pairs (Buckner et al., 2000; Gold et al., 1993).

The Connection Between Cotton Fibers and Tracheary Elements

It has been proposed that cotton fiber maturation is similar to tracheary element differentiation and involves programmed cell death (Kim and Triplett, 2001). Tracheary elements, the cells composing xylem vessels, are characterized by secondary cell wall formation, cytoskeleton reorganization, deposition of cellulose, and programmed cell death.

Secondary wall formation and programmed cell death are thought to be coordinated in *Zinnia elegans* (zinnia), where, much like cotton fibers, the final product of tracheary element differentiation and maturation is a cell corpse composed of secondary cell wall (Motosé et al., 2004; Groover and Jones, 1999; Potikha et al., 1999). The elaters of *Symphyogyna* (liverworts) and *Megaceros* (hornworts), a lesser-known cell type, are strikingly similar to cotton fibers and tracheary elements. They too undergo cell elongation and secondary cell wall formation, with maturation characterized by drying, twisting, and death (Kremer and Drinnan, 2003). They also have arabinogalactan proteins present in their secondary cell walls (Kremer et al., 2004).

Tracheary elements are proposed to initiate a programmed cell death cascade succeeding rupture of the large central vacuole, likely releasing caspases. Cotton fibers too, have a large central vacuole comprising most of the intracellular space. There have yet to be reports published which indicate vacuole rupture in cotton fibers. As fiber cells dehydrate between 45 and 60 DPA, the vacuole does disappear as the cytoplasm dries up, leaving a lumen in its place (Kim and Triplett, 2001). The central vacuole has been shown to be intact in 40 DPA *in vitro* cultured fibers (Westafer and Brown, 1976). However, cotton fibers develop at a much slower rate *in vitro* than *in vivo* (Meinert and Delmer, 1977). The actual point of vacuole rupture, if it occurs in this system, may occur at or prior to 40 DPA in plant-grown fibers.

Role of Hydrogen Peroxide and Other Reactive Oxygen Species in Cell Elongation, Secondary Wall Synthesis and PCD

Hydroxyl (OH), a reactive oxygen species (ROS), is implicated in the remodeling of xyloglucan, leading to cell elongation (Demidchik et al., 2003). Xyloglucan, involved in microfibril linking, is highly expressed during the elongation period of cotton fibers (Cosgrove, 1999; Tokumoto et al., 2002).

A vacuolar H⁺-ATPase catalytic subunit is shown to accumulate more than 50 fold in 10 DPA fibers compared to 0 DPA ovules, with the greatest accumulation between 5 and 15 DPA (Ji et al., 2003; Smart et al., 1998). Activation of H⁺-ATPase is implicated in inducing hydrogen peroxide production (Malerba et al., 2004) and in acidification of the apoplast, leading to cell wall loosening and thus, elongation (Rober-Kleber et al., 2003).

Hydrogen peroxide (H₂O₂), noted for its ability to cause cell damage and cell death, has been detected in cotton fiber cells undergoing secondary cell wall synthesis (16-20 DPA) by Potikha et al. (1999). The group proved its role in the differentiation of the secondary cell wall by removing H₂O₂ from the system. This action prevented synthesis of secondary walls. They further proved its action by treating young fibers with H₂O₂, and observing the subsequent premature differentiation of cell walls.

Rac13, an activator of NADPH oxidase, is a highly active gene at the onset of secondary cell wall synthesis (15-18 DPA) in cotton fibers. This activation leads to an oxidative burst, increasing cellular levels of ROS (Delmer et al., 1995). Prolonged exposure to ROS (O₂⁻, H₂O₂, OH) causes stress in the cell, which can lead to programmed cell death. *Rac13* expression declines after secondary cell wall synthesis begins (Delmer et al., 1995). As such, H₂O₂ content declines as well (Potikha et al., 1999).

Typically, high levels of H₂O₂ (8 mM) are required in order to trigger PCD (Levine et al., 1996). However, it has been proposed (Potikha et al., 1999) that although the concentration of H₂O₂ in cotton fibers is much lower (5 μM/mg d.w.), the exposure

occurs over a period of several days, thus possibly inciting the same effect as higher concentrations. Houot et al. (2001) induced PCD in cultured tobacco BY-2 cells by addition of H₂O₂ to the culture medium. Although the concentrations used were much higher (12.5 mM) than those reported in cotton fibers, the results indicate that longer incubation times with lower concentrations of H₂O₂, produced higher levels of programmed cell death.

Hydrogen peroxide accumulates in plant cell walls during the hypersensitive response (Grant et al., 2000). A minimum of 4 – 6 mM sustained H₂O₂ in cells is required to initiate hypersensitive cell death (Levine et al., 1996). As the concentration in cotton fibers is much lower, it is not likely that HR-type cascade occurs here. The programmed cell death of xylem tracheary elements is not initiated by an oxidative burst (Groover et al., 1997). However, a role for ROS in the death of these cell types is possible.

Role of Arabinogalactan Proteins in Secondary Wall Formation and PCD

Arabinogalactan proteins (AGPs) are proteoglycans that play a role in plant growth and development (Schultz et al., 2000). AGPs are composed of a hydroxyproline-rich core decorated with arabinose, galactose and other less abundant polysaccharides, and are primarily located in plasma membranes, cell walls and intracellular space (Showalter, 2001). Arabinogalactans are thought to have roles in cell elongation, cell signaling, and programmed cell death. AGPs, present in both tracheary elements and cotton fibers, are hypothesized to mark cells destined for PCD; a conclusion based on the highly regulated AGP expression during xylem development (Majewska-Sawka and Nothnagel, 2000).

AGP (*PtaAGP6*) labeling in loblolly pine by anti-AGP antibody JIM13, is restricted to cells just entering secondary cell wall development and those about to begin differentiation (Zhang et al., 2003). In the maize-coleoptile, AGPs are found localized to the plasma membrane and cell wall of cells destined to differentiate into tracheids and schlerenchyma, both processes which require PCD (Buckner et al., 2000), indicating

they play a role in tracheary element formation (Gao and Showalter, 1999). JIM13 and JIM14 antibodies only recognized disintegrating cells in the vascular region of maize coleoptiles (Schindler et al., 1995). In zinnia mesophyll cells, AGPs are thought to act as inducers of tracheary element differentiation, a process culminating in programmed cell death (Chaves et al. 2002). Xylogen, an AGP-lipid transfer protein hybrid, is required for xylem differentiation in zinnia mesophyll cells, and localizes to cell walls undergoing tracheary element differentiation (Motosé et al., 2004). *LeAGP-1* was localized to the differentiating xylem and metaxylem in stems and roots of tomato plants from initiation to completion (Gao and Showalter, 2000). Overexpression of *LeAGP-1* in tomato produced stunting in later stages of growth, purportedly related to interference of secondary cell wall synthesis. The leaves of these plants also showed delayed senescence (Sun et al., 2004a).

Differentiating xylem and developing cotton fibers show similarity in that both undergo cell elongation and secondary cell wall synthesis, concluding in cell death. *Ptx14A9*, an AGP identified in poplar, preferentially expressed in differentiating xylem, has an ortholog in cotton and is expressed in fibers. (Yang et al., 2005). The cotton E6 protein has been detected in fibers throughout development and localizes to primary and secondary cell walls of cotton fibers. The highest expression of E6 is noted during the transition from primary to secondary cell wall stages (John and Crow, 1992). E6 shares homology with the Asn-rich domain of non-classical AGPs (Gaspar et al., 2001). The cotton H6 protein, thought to be an arabinogalactan protein, is present in the cell walls during fiber elongation beginning at 15 DPA and is found to accumulate during secondary cell wall synthesis until 30 DPA, declining thereafter (John and Keller, 1995).

Arabinogalactans are known to be present in the cell wall of cotton fibers. Specifically, AGP mRNAs (AGP1, AGP2 and AGP4) have been found to significantly accumulate until 10 DPA, at which point a plateau is reached and a reduction occurs thereafter (Feng et al., 2004; Ji et al., 2003). AGPs are known to be involved in cell elongation and their disruption from the plasma membrane causes cell growth arrest and Ca^{2+} release, leading to H_2O_2 production and eventually PCD (Chaves et al., 2002).

AGPs are specifically bound by β -galactosyl Yariv reagent. Depending on concentration they may be stained or precipitated from the plasma membrane. When cotton fibers are stained with β -galactosyl Yariv reagent, maximal staining is noted during the period of secondary wall deposition, while lesser staining is observed during elongation. However, it is believed that the cellulose in cotton fibers interferes with the binding of β -galactosyl Yariv reagent to AGPs (Triplett and Timpa, 1997). Precipitation of AGPs inhibits both cell elongation and cellulose deposition, in addition to causing cell death in *Nicotiana tabacum* (tobacco) (Vissenberg et al., 2001; Chaves et al., 2002). It is believed their perturbation interferes with cell wall assembly and inhibits expansion (Majewska-Sawka and Nothnagel, 2000).

Arabinose, galactose, rhamnose, fucose, xylose and mannose, are the sugars comprising arabinogalactans (Table 1). AGPs are highly soluble and their sugars are shown to be preferentially released from the plasma membrane during secondary cell wall formation in cotton fiber (Table 2) (Meinert & Delmer, 1977). Arabinose and galactose were also noted in the soluble fractions of fiber cell walls predominantly at 10 DPA, indicating the presence of AGPs (Maltby et al., 1979). Autolysis of primary cell walls, causing release of carbohydrates, was observed in zinnia cells during secondary cell wall synthesis, with release of the same sugars as in cotton (Ohdaira et al., 2002).

AGPs may be disintegrated by treatment with glycosidases (e.g. by β -galactosidase or α -L-arabinofuranosidases) (Showalter, 2001). Treatment of human T cells with galactin-1, a β -galactoside binding protein, induces apoptosis (Pennell and Lamb, 1997). *GhGall*, a β -galactosidase, is preferentially expressed in cotton fibers during the elongation phase, with a huge decline in expression noted upon entry into secondary wall synthesis (20 DPA). *GhGall* has D-galactose and L-rhamnose binding domains, indicating its role in the turnover of galactose containing polysaccharides, (Zhang and Liu, 2005) likely arabinogalactans (Aspeborg et al., 2005). A putative β -galactosidase is also highly down-regulated in 24 DPA over 10 DPA cotton fibers (Arpat et al., 2004). α -L-arabinofuranosidases are enzymes acting to release arabinose from arabinogalactans among other substrates. These enzymes are shown to be active in the

secondary wall phase of aspen (*Populus tremula x tremuloides*), likely acting in the modification of AGPs (Aspeborg et al., 2005) and are shown to release 90% of the arabinose from isolated cotton fiber arabinogalactans (Buchala and Meier, 1981). It is thus likely that glycosidases are responsible for the release of AGP sugars from the plasma membrane in cotton fibers.

Table 1. Sugar Composition of
A. thaliana Root Arabinogalactans

Sugar	Molecular %
Galactose	44.7 (1.8)
Arabinose	27.0 (0.9)
Glucose	13.0 (3.0)
Fucose	4.2 (0.2)
Rhamnose	3.0 (0.3)
Xylose	2.9 (0.1)
Mannose	1.5 (0.1)
Uronic acids	3.7 (0.2)

Standard errors in brackets. Adapted from Van Hengel and Roberts, 2002.

Table 2. Neutral Sugar Composition of Fiber Cell Walls at Various Ages

Sugar	Age of Fiber								
	5	10	14	15	16	18	22	25	29
Rhamnose	5.9 (0.6)	7.4 (0.9)	8.4 (0.5)	6.7 (0.4)	4.3 (0.2)	5.1 (0.0)	2.3 (0.2)	3.1	1.7 (0.3)
Fucose	0.9 (0.3)	1.2 (0.7)	1.2 (0.3)	1.0 (0.7)	0.4 (0.4)	0.5 (0.6)	0.4 (0.1)	0.2	0.3 (0.0)
Arabinose	24.4 (2.6)	32.8 (3.1)	22.3 (2.1)	13.6 (0.2)	7.9 (0.6)	8.6 (1.3)	4.1 (0.1)	5.6	5.5 (0.2)
Xylose	7.7 (0.5)	10.2 (1.4)	10.3 (0.4)	9.0 (0.3)	6.6 (0.4)	8.5 (0.6)	4.0 (1.0)	3.7	3.5 (0.1)
Mannose	3.5 (0.8)	3.9 (0.3)	3.9 (0.3)	3.6 (0.6)	1.7 (0.5)	1.9 (0.6)	2.5 (1.6)	1.3	1.2 (0.1)
Galactose	12.9 (0.7)	17.1 (1.9)	17.8 (0.3)	13.4 (0.7)	7.4 (0.8)	8.4 (0.0)	4.5 (1.2)	5.9	4.9 (0.1)
Glucose	45.0 (2.0)	28.0 (6.3)	36.2 (2.5)	52.7 (0.7)	71.7(0.9)	67.1 (0.9)	82.4 (3.6)	80.4	83.0 (0.0)
Uronic Acids	10	22	16	9		3	2	0	0
Total %	32.4 (2.3)	23.3 (2.1)	21.0 (2.2)	13.8 (2.3)	13.8 (3.5)	10.0 (1.1)	10.1 (0.1)	10	5.8 (0.2)

Each sugar expressed as mole percent of the total sugars. Standard errors in brackets.

Total neutral sugar content determined by GLC. Adapted from Meinert & Delmer, 1977.

The changes occurring in fiber cell wall neutral sugar content parallel that of arabinogalactan content, all showing an increase until just prior to the onset of secondary wall formation. In addition to neutral sugars, uronic acid also followed the pattern of increase until secondary wall synthesis, followed by a great decline, indicating its complete removal from the cell wall (Meinert and Delmer, 1977). Of the uronic acids released from cell walls in differentiating zinnia cells, 82% were found to be polygalacturonic acid, the remainder being glucuronic acid (Ohdaira et al., 2002). This is interesting in light of the fact that polygalacturonic acid induces H_2O_2 production, an inducer of secondary wall synthesis, which begins at approximately the same time as uronic acid removal. Oligogalacturonic acid in tobacco leaf explants has shown to stimulate cellulose deposition (Potikha et al., 1999). Another factor surrounding the changes in cell wall polysaccharides is the release of D-mannose. D-mannose treatment has been shown to cause PCD in arabidopsis roots and maize suspension-cultured cells (Stein and Hansen, 1999).

As AGP perturbation by chemical means (β -glucosyl Yariv reagent) has been shown to cause induction of PCD, it can be postulated that release of arabinogalactan carbohydrate moieties signals the onset of secondary wall synthesis and PCD in the cotton fiber through oligosaccharide chemical signaling (Showalter, 2001). It is likely that AGPs play a role in elongation of the cotton fiber cell and are released from the plasma membrane in order to signal secondary wall formation and maturation.

Role of Brassinosteroids in Elongation, Secondary Wall Differentiation and PCD

Brassinosteroids (BR) directly regulate xyloglucan endotransferases and are known to play roles in fiber cell initiation and elongation (Klahre et al., 1998; Sun et al., 2004b). They are also known to directly upregulate expression of AGPs. Cytochrome p450s are involved in BR biosynthesis and exposure of cells to cytochrome p450s generates ROS and is thought to play a role in programmed cell death (Sun et al., 2005; Gonzalez,

2005). A cytochrome p450-like protein was found to accumulate greater than 280-fold in 10 DPA vs. 0 DPA ovules (Ji et al., 2003). Xylem differentiation and subsequent PCD are dependent on brassinosteroids, and BRs are specifically initiated during the transition from secondary cell wall synthesis to PCD (Yamamoto et al., 2007). In an array of 24 DPA vs. 10 DPA fibers, brassinosteroid biosynthetic protein LKB is highly down-regulated (Arpat et al., 2004). This protein shows very high homology to the *Diminuto/Dwarf1* gene of arabidopsis.

The *Diminuto/Dwarf1* gene of arabidopsis, a cell elongation factor, is homologous to *GhFe1* in cotton and the *Seladin-1* gene in humans. *Seladin-1* confers resistance to Alzheimer's Disease, an affliction characterized by apoptotic death of brain cells. It is believed this gene aids in protection against oxidative stress (Greeve et al., 2000). The *diminuto* mutant has a reduced AGP and BR content, although the reduction in AGP is likely not a direct affect of the mutation (Schultz et al., 1998; Schultz et al., 2000; Klahre et al., 1998). *ZeDIM* of zinnia, is most highly expressed during secondary cell wall synthesis and PCD (Yamamoto et al., 2007). Arabidopsis *dwarf1* mutants have both reduced brassinosteroid and arabinogalactan content. These mutants have short stature and extended life spans (Choe et al., 1999).

Role of Calcium and Callose in Elongation, Secondary Wall Synthesis and PCD

Callose (β -1,3-glucan) deposition at the plasmodesmata and wound sites is a common feature of the oxidative burst; a hypersensitive stress response leading to PCD. Presence of callose in cells has served as an indicator of cells destined to undergo PCD (Blackman and Overall, 2001; Broderson et al., 2002). In cotton fibers, callose and cellulose are synthesized by sucrose synthase (SuSy). Callose forms during the elongation phase and at the onset of secondary wall synthesis (Amor et al., 1995). Closure of the plasmodesmata into the fiber cell, presumably by callose, occurs during the phase of rapid cell elongation (10 – 16 DPA) (Ruan et al., 2001). The callose at the fiber base is later degraded. A second deposition of callose is noted at the onset of secondary cell

wall development and continues throughout this stage, after which a switch to cellulose synthesis occurs (Ruan et al., 2004; Salnikov et al., 2003; Amor et al., 1995).

Callose formation requires an influx of Ca^{2+} (Andrawis et al., 1993) and it is thought that SuSy only produces callose when Ca^{2+} predominates, otherwise cellulose is produced (Amor et al., 1995). A thin layer of callose at the innermost layer flanking the cell lumen has been noted in fibers with secondary walls until the completion of fiber development (Salnikov et al., 2003 and Waterkeyn, 1981). Mature cotton fiber cell walls are composed nearly entirely of cellulose (95%) (Kim and Triplett, 2001) and callose has been reported at 0.1-0.3% (Maltby et al., 1979). SuSy also immunolocalizes at the cell plate of differentiating xylem tracheary elements during secondary wall deposition, indicating the presence of callose (Amor et al., 1995). The callose depositions in cotton fiber are developmental and patterned and are not to be confused with wound callose, which is irregular in wounded cells (Waterkeyn, 1981).

An increased expression of H^+ -ATPase occurs at the time of plasmodesmatal closure in cotton fibers, causing an increase in ROS (Ruan et al., 2004). Free oxygen radicals lead to increases in cytosolic Ca^{2+} levels. Root growth accelerates significantly with slight increases in cytosolic Ca^{2+} (Demidchik et al., 2003). This activation was significant for arabidopsis root cells undergoing elongation. As quenching of free radicals inhibited this root elongation, Ca^{2+} influx is shown to have a causal role in cell elongation. Cotton fiber cells as well, show a greater than 50-fold accumulation of calmodulin, a Ca^{2+} binding protein, in 10 DPA compared to 0 DPA ovules (Ji et al., 2003). There is also an influx of Ca^{2+} in cotton fibers during the transition from primary to secondary wall formation. Ca^{2+} has been shown to play a role in activation of NADPH oxidase, indicating a role in secondary wall formation (Potikha et al., 1999). Ca^{2+} influx is known as a requirement for apoptosis in both plant and animal cells (Groover and Jones, 1999). Ca^{2+} is required for PCD in tracheary elements, as it triggers mitochondrion to undergo a permeability transition, releasing apoptosis proteins into the cytosol, such as cytochrome c and procaspases (Yu et al., 2002).

Experimental Rationale

Arabidopsis cells undergoing PCD, showed greater than a 3.8 fold upregulation in extensin (*EXT1*), cytochrome P450 (*PAD3*), calmodulin (*TCH3*) and calmodulin-like genes, among others (Brodersen et al., 2002). Microarray analysis of *A. thaliana* leaves undergoing senescence showed a greater than three-fold upregulation in over 800 genes including calmodulin, β -1,3-glucanase, plasma membrane ATPase, arabinogalactan (*AGP5*), xyloglucan fucosyltransferase (*FUT6*), and cytochrome p450s (Buchanan-Wollaston et al., 2005). The comparable expression of these genes indicates that PCD is a highly regulated process, requiring the activation of many interrelated pathways that are connected to the developmental phases of elongation and secondary wall synthesis.

Very little is known about mature cotton fibers due to the difficulty in extracting nucleic acids after cellulose has been deposited. Cotton fiber DNA extraction much past 25 DPA may not be achieved by typical methods, i.e. grinding with mortar and pestle, due to the presence of the secondary wall (Wilkins and Arpat, 2005). As PCD appears to be occurring past this time point, it was fitting to attempt DNA extraction by another method. As nuclei extraction methods for cotton had already proven successful, I attempted to extract nuclei from cotton fiber at set 5 DPA increments and used the extracted nuclei for DNA isolation, followed by visualization with agarose gel.

It is known that cotton fiber cells die at some point in their development, but the means is unknown, although PCD is hypothesized to be the cause. Necrosis, the other type of cellular death, typically occurs due to direct injury to the cell surface. Necrosis, in contrast to PCD, is not an active and gene dependent form of death (Wang et al., 1996). Necrotic cells may be characterized initially by swelling and lysis of the plasma membrane (Chaves et al., 2002) and eventually, necrotic cells will show nuclear swelling and cell lysing. (Buckner et al., 2000). A notable difference is that necrotic cells will have a random fragmentation of DNA, indicated by a smear on an agarose electrophoresis gel; as such, necrotic cells typically will not stain positively for terminal deoxynucleotidyl transferase (TdT) mediated dUTP nick end labeling (TUNEL). TUNEL utilizes the TdT enzyme in order to add dUTP fluorescein to free 3' OH ends

produced when DNA is cleaved during apoptotic, but not necrotic cell death (Exley et al., 1999). This method was utilized to determine by what means and at what point in cotton fiber development, death was occurring.

To date there have been no reports showing any molecular or biochemical markers for programmed cell death in the maturing cotton fiber (Kim and Triplett, 2001). Models for the coordination of secondary cell wall synthesis and PCD have been proposed previously (Groover and Jones, 1999). The formation of secondary cell walls in cotton fibers has been described extensively. Although examinations of fiber DNA and PCD are inconclusive in this study, the time course of secondary cell wall formation and maturation may be observed, in addition to intriguing patterns of PI and TUNEL staining throughout development.

RESULTS

Fibers were collected from greenhouse grown *G. hirsutum* at intervals of 5 DPA, from 5 through 65 DPA. Each time-point was sampled at least twice and multiple DPAs were collected on a given day. Results were consistent across sampling dates. Fibers were subsequently used for nuclear and DNA extractions, and TUNEL and propidium iodide (PI) staining and visualization.

DNA Ladder Gel

Nuclei isolated from fibers at the time points 5, 20, 25, 30, 35, 40, 45, 50, 55, 60, and 65 DPA were used for DNA extraction using the Roche Apoptotic DNA Ladder Kit and extracted DNA was subjected to agarose gel electrophoresis. Initial attempts to visualize DNA using 6 μ L DNA per lane and in-gel ethidium bromide (EtBr) staining proved unsuccessful. DNA became visible when a 20 μ L sample was loaded per lane and, in addition to in-gel staining with EtBr, subsequent staining with SYBR Gold was performed. This method still only proved successful for DNA extracted from fibers up to 40 DPA. No DNA was visible at later stages (results not shown). At no stage of development was a DNA ladder observed (Figure 1).

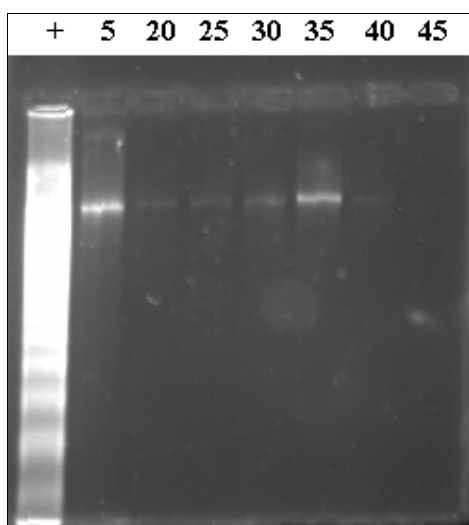


Figure 1. Agarose Gel Electrophoresis of Isolated Cotton Fiber DNA.

In-gel stained with ethidium bromide and then stained with SYBR Gold for 1hr after electrophoresis. Lane 1 represents positive control apoptotic U937 lymphophilized cells. Lane numbers represent fiber age in days post anthesis (DPA). 5 and 35 DPA lanes contain fiber and ovule tissue DNA. Note that no band is visible for 45 DPA and no laddering is apparent at any point.

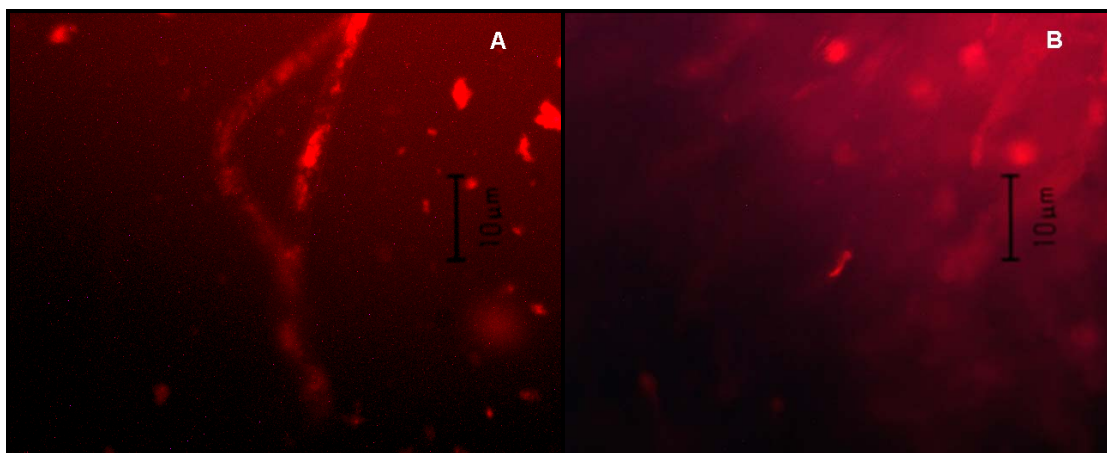


Figure 2. 5 DPA Cotton Fiber and Ovule Tissue.

(A) 5 DPA fiber exposed for both fluorescein (TUNEL) and PI (DNA). Stained ovule present in the background. (B) 5 DPA ovule tissue with fibers extending from it. Exposed for both PI and fluorescein.

TUNEL Assay

Fibers were collected in five-day increments between 5 and 65 DPA. These fibers were stained using the TUNEL assay to detect DNA strand breaks and by PI to indicate the presence of DNA. Samples were viewed with a fluorescence microscope under ultraviolet (UV) excitation for fluorescein dUTP and green excitation for PI, unless otherwise noted. All samples were stained with both fluorescein dUTP and PI.

At 5 DPA, ovule tissue and fibers were collected, due to the small mass of fibers at this stage of development. Fibers at this stage were TUNEL-negative. In fibers at this and late stages, the PI signal extended through the fiber's length (Figure 2A). Ovule cells, however, contained conventionally shaped nuclei (Figure 2B). A similar pattern of fiber staining was observed at 10 DPA (Figure 3) and 15 DPA (Figure 4). The cell wall appeared thin in 15 DPA fiber images compared to 20 DPA, indicating they were pre-formation of secondary cell wall. It is likely that differentiation does not begin until at least 16 DPA. The faint autofluorescence of primary cell walls may be noted in elongation stage fibers (5-15 DPA).

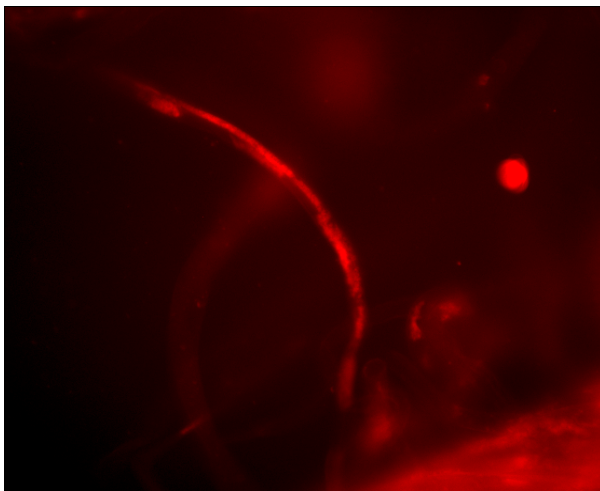


Figure 3. 10 DPA Cotton Fiber.

10 DPA fibers exposed for PI.

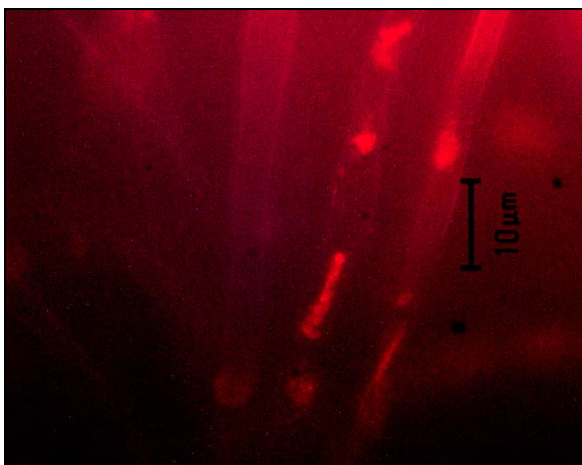


Figure 4. 15 DPA Cotton Fiber.

Exposed for PI (DNA) and fluorescein (TUNEL). Note fluorescence of the thin primary cell wall.

The first sign of secondary wall thickening can be observed in 20 DPA fibers. Thickening sections of cell wall were TUNEL-positive, although no TUNEL signal derived from within the cell (Figure 5). The PI emission at this stage appears through the length of the cell.

The first observation of intracellular TUNEL staining was made in 25 DPA fibers (Figure 6). Condensation of PI stained material occurs at this stage, as well. A preference for fluorescein staining of cell wall sections, presumably in the secondary wall synthesis stage, can be noted by the different staining patterns between Figure 6A and B. The cell walls in TUNEL-positive fibers are noticeably thicker, indicating these cells are synthesizing secondary wall.

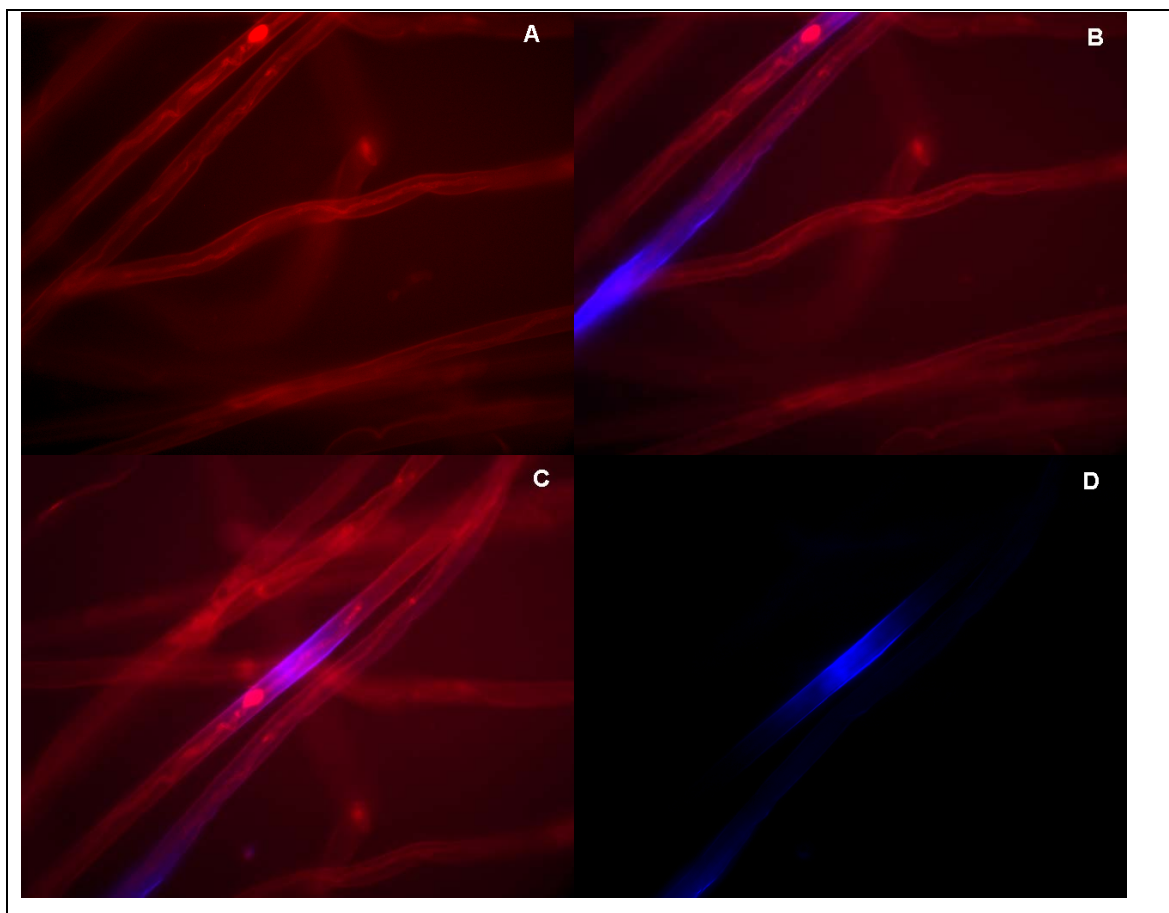


Figure 5. Cotton Fibers at 20 DPA.

The same section appears in **(A)** and **(B)**. **(A)** Exposed for PI (DNA). Note elongate appearance of PI stained area. **(B)** Exposed for PI and fluorescein (TUNEL). Note TUNEL-positive cell wall sections. **(C)** and **(D)** are the same section. **(C)** Exposed for PI and fluorescein. **(D)** Exposed for only fluorescein. Note the thickened TUNEL-positive cell wall section, possibly indicating secondary wall synthesis is occurring there.

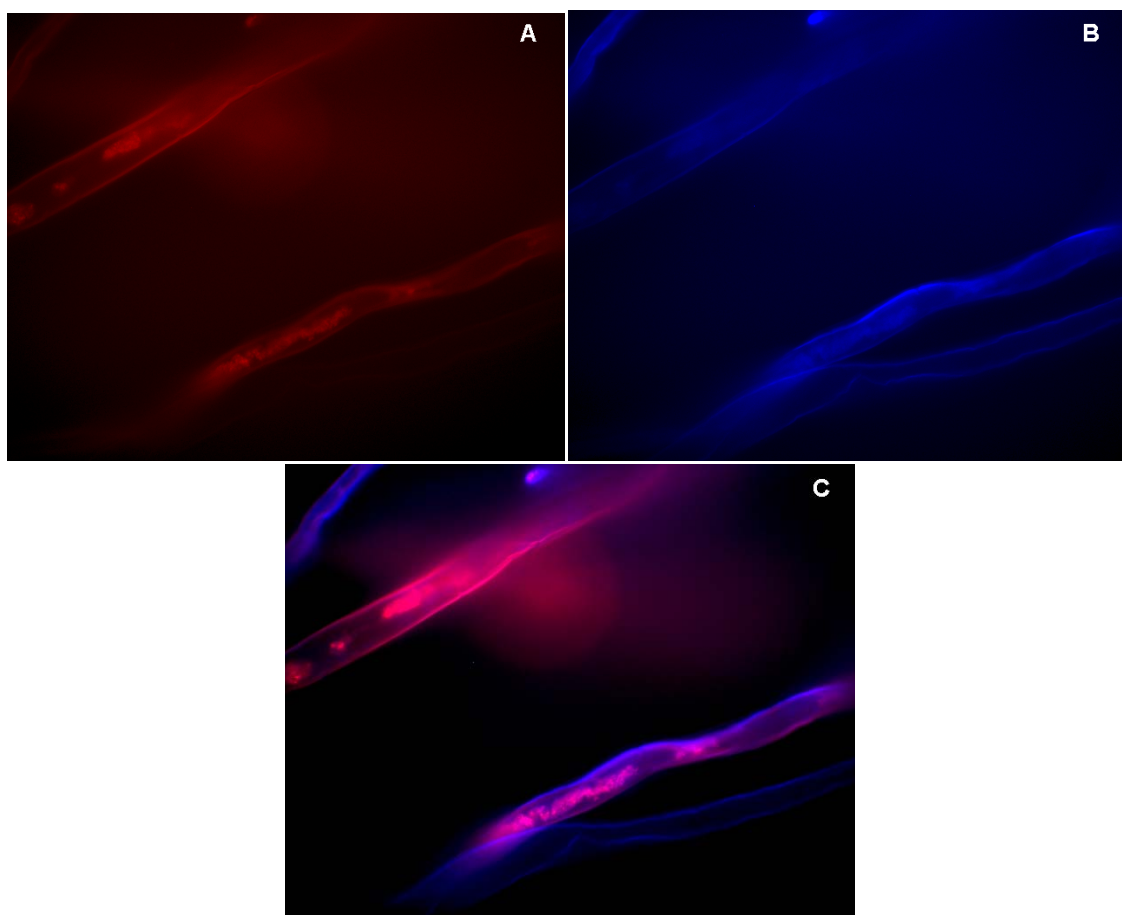


Figure 6. Cotton Fibers at 25 DPA

Same section in all images. **(A)** Exposed for PI to detect DNA. **(B)** Exposed for fluorescein (TUNEL). **(C)** Exposed for PI and fluorescein. Note the different staining patterns in individual fibers.

The different staining patterns in 30 DPA fibers (Figure 7A) indicate they are at different stages of development. Some fibers are TUNEL-positive, while others remain TUNEL-negative. At this point, a condensed TUNEL signal may be observed (Figure 7B). By 35 DPA, the TUNEL-positive emission appears further condensed and becomes more widespread across fiber samples (Figure 8).

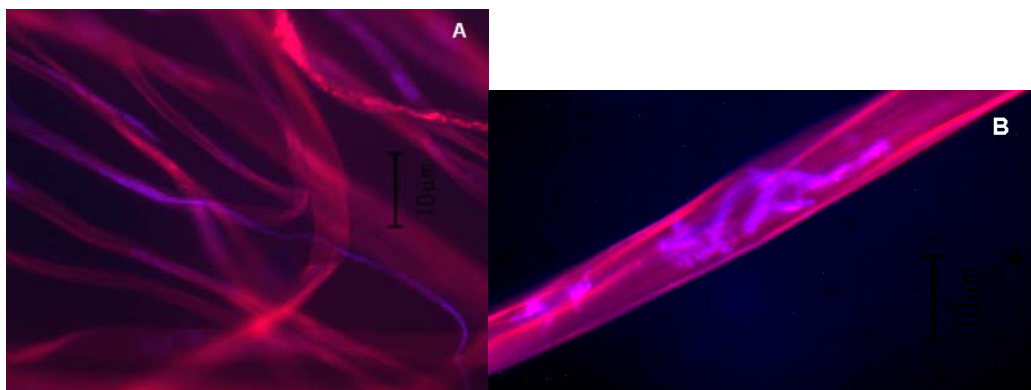


Figure 7. 30 DPA Cotton Fibers.

(A) Note some fibers are TUNEL-positive and some are TUNEL-negative. (B) Close up of a TUNEL-positive fiber section. Both images exposed for PI (DNA) and fluorescein (TUNEL).

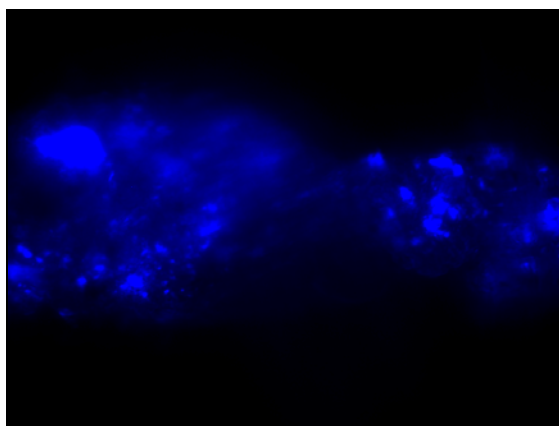


Figure 8. 35 DPA Cotton Fiber.

Exposed for fluorescein (TUNEL). Note condensed TUNEL-positive signal.

By 40 DPA, fibers feature condensed TUNEL-positive areas and thickened cell walls (Figure 9). After storage for approximately 10 months at -70°C , 40 DPA fibers

were stained with PI and fluorescein dUTP to determine the DNA longevity in stored cells. There was a complete lack of visible staining in these cells.

Fibers at 45 days post-anthesis contained highly condensed, TUNEL-positive portions (Figure 10). All captured images of 45 DPA fibers were TUNEL-positive. If fluorescein is actually staining fragmented DNA, the images at this point indicate that this is the time-point at which PCD is most widespread in the cotton boll. It may also be noted at this stage that fluorescein may be excited by both UV and blue light, as in Figure 10C and D, it can be observed that the pattern of staining is nearly identical. The TUNEL-positive area has an unusual dot-dash pattern. The unstained areas may be organelles or small vacuoles.

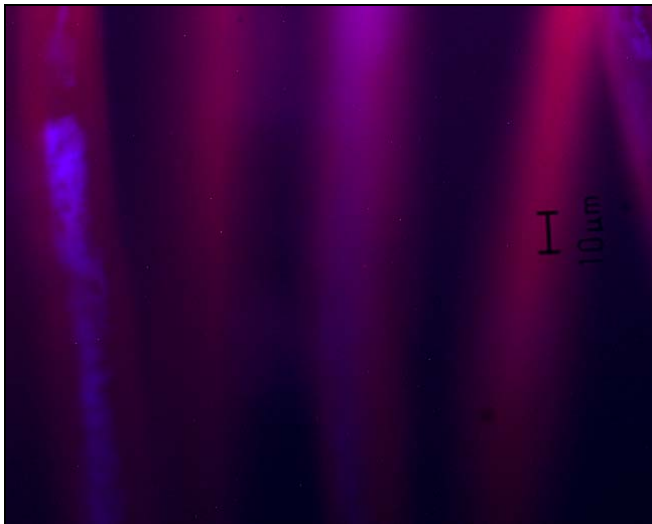


Figure 9. 40 DPA Cotton Fibers.

Exposed for PI (DNA) and fluorescein (TUNEL). Note different degree of TUNEL signal in each fiber cell.

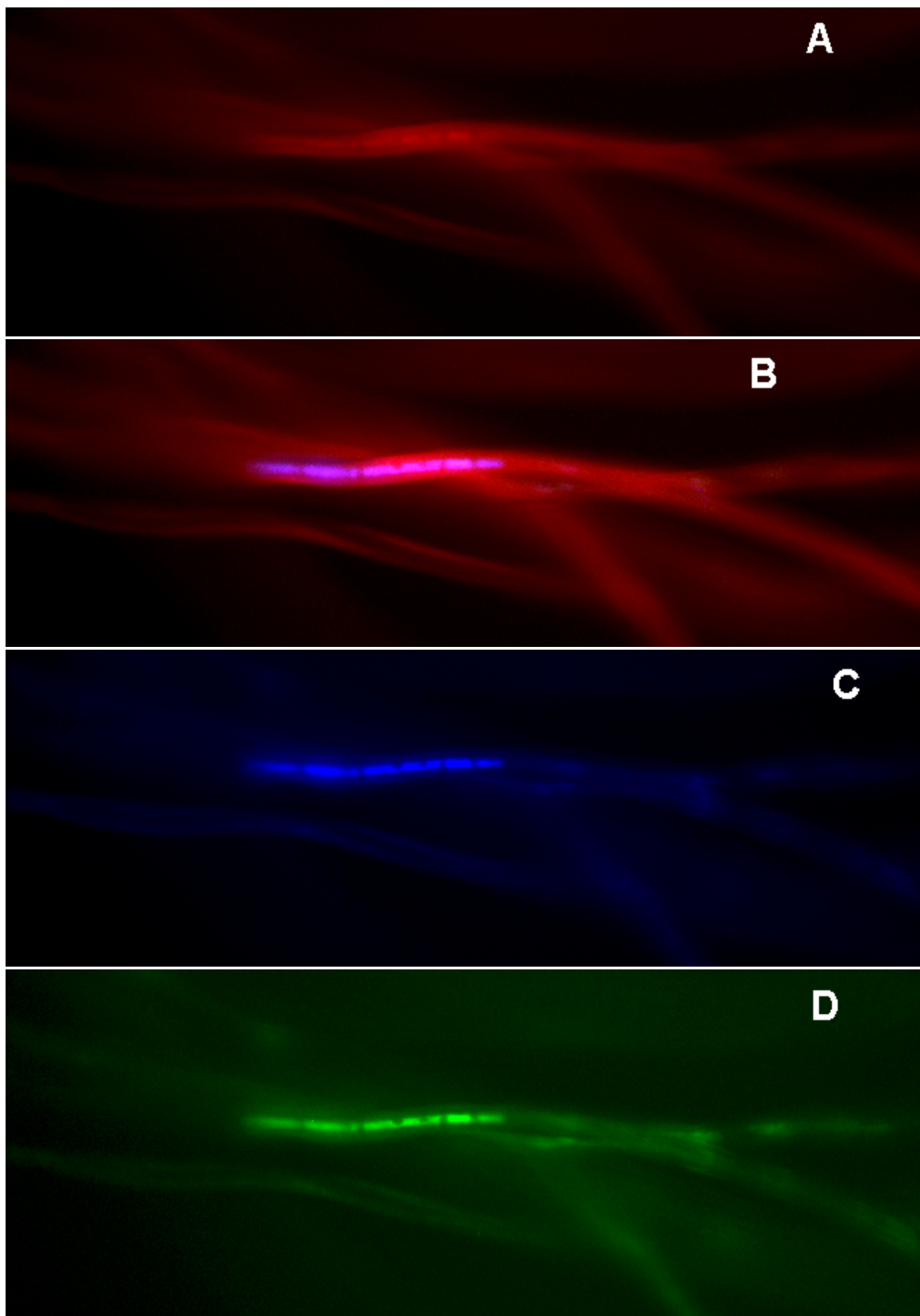


Figure 10. 45 DPA Cotton Fibers.

The same section appears in each image. **(A)** Exposed for PI (DNA). **(B)** Exposed for PI and fluorescein (TUNEL). **(C)** Exposed for fluorescein. **(D)** Exposed under blue light for fluorescein. Note the same sections fluoresce in both **(C)** and **(D)**.

By 50 DPA, the TUNEL signal begins distribute throughout the cell (Figure 11). Also, twisting of the fiber becomes observable, due to the drying out that occurs during fiber maturation. The secondary cell wall is prominent in this stage, being highly stained by PI. By 55 DPA, a definite separation in TUNEL emitting sections can be observed (Figure 12B). A 55 DPA image exposed for only PI shows that the intracellular space is no longer stained, as only the cell wall fluoresces red at this time point (Figure 12A). However, the TUNEL-positive signal remains.

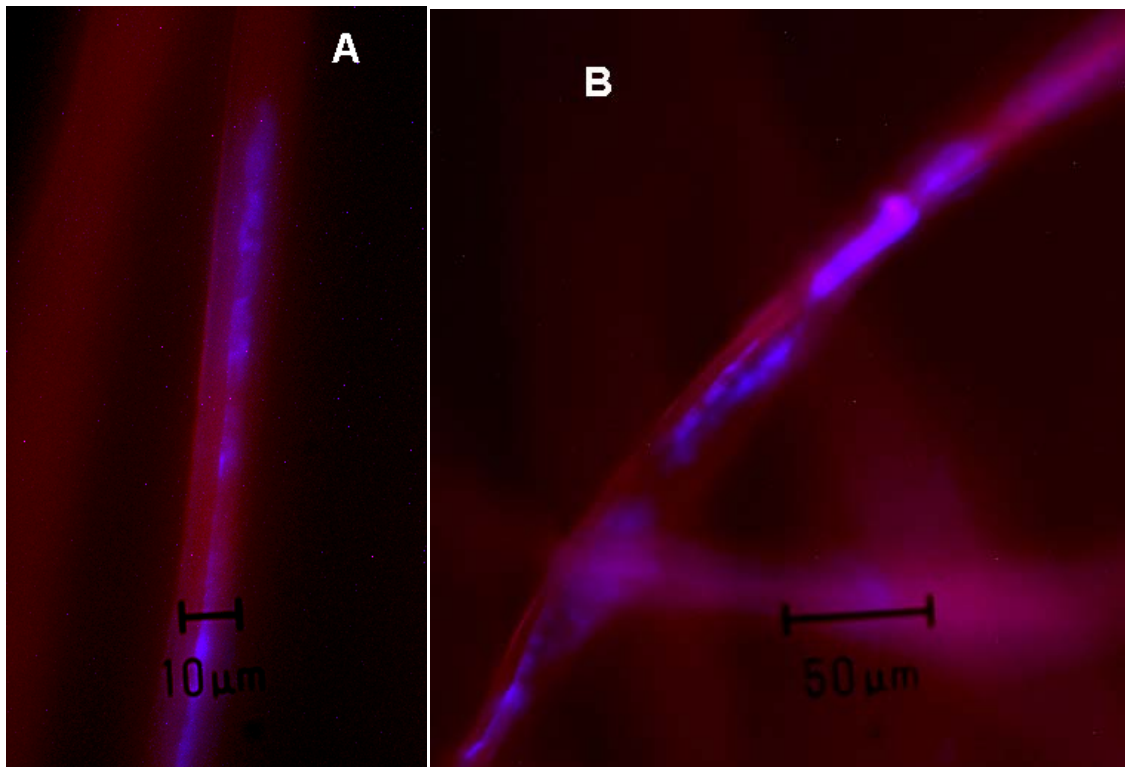


Figure 11. 50 DPA Cotton Fibers.

Examples of TUNEL-positive staining in 50 DPA fibers. Both exposed for PI (DNA) and fluorescein (TUNEL). **(A)** Note highly thickened cell walls. **(B)** Note twisting of fiber.

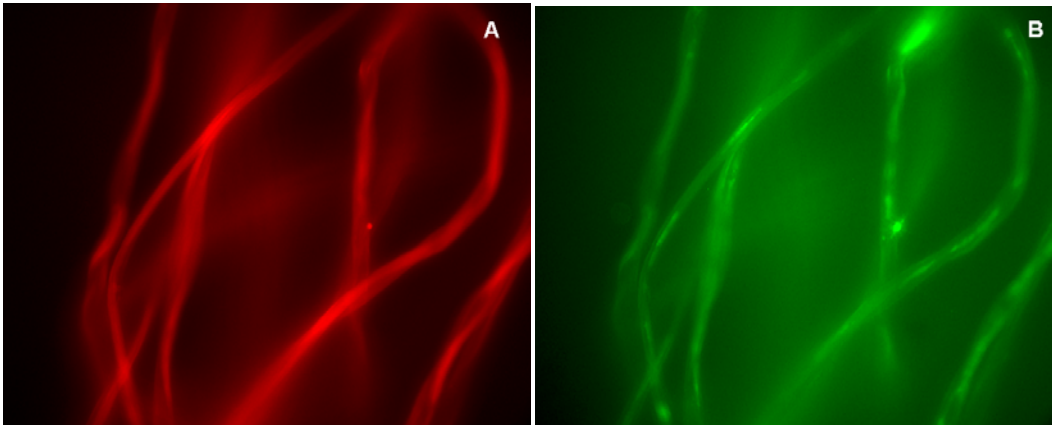


Figure 12. 55 DPA Cotton Fibers.

Same section of fibers in **(A)** and **(B)**. **(A)** Exposed for PI (DNA). Note only the cell walls fluoresce. **(B)** Exposed under blue light for fluorescein (TUNEL). Note the TUNEL-positive sections appear separated.

In 60 DPA fibers, the TUNEL signal became more dispersed (Figure 13). The fibers at this developmental stage were more twisted, as they were almost completely, if not completely, dried out. Minimal intracellular PI staining may be observed in these fibers as well (Figure 13A). Fibers at 65 DPA appear to be completely dehydrated and twisted (Figure 14A). The secondary cell walls comprise nearly the entire cell volume, with only a narrow TUNEL-positive area visible (Figure 14B-D).

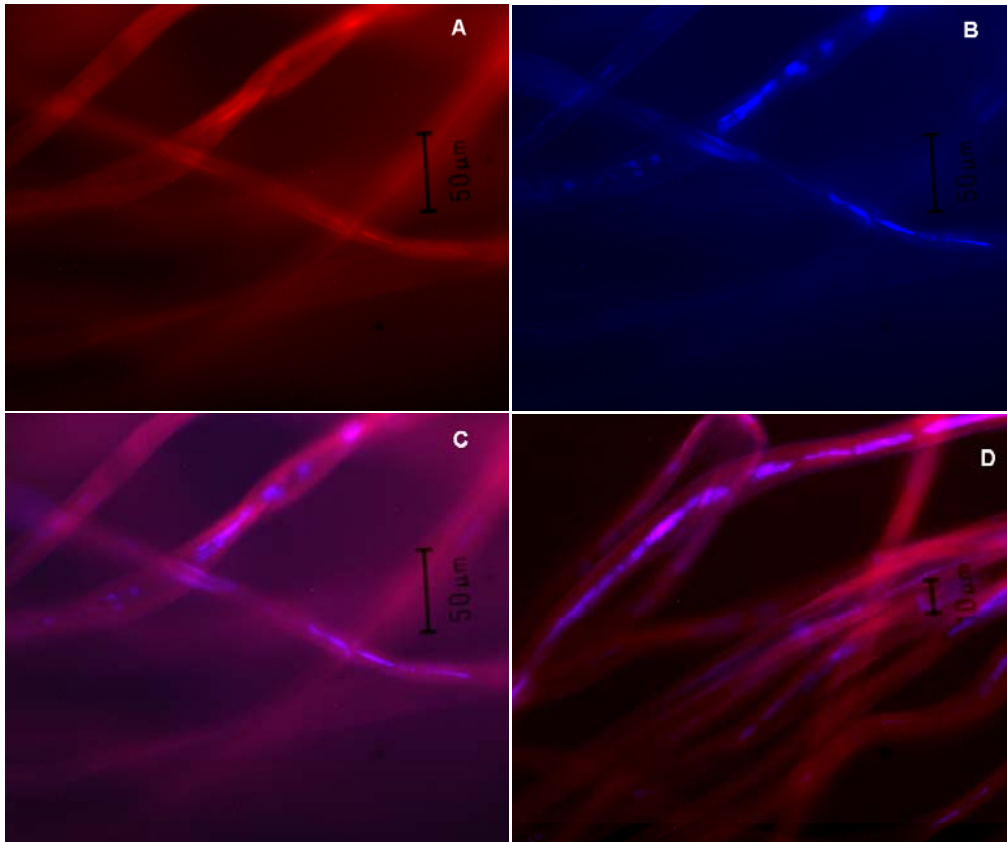


Figure 13. 60 DPA Cotton Fibers.

Same fiber section appears in (A), (B) and (C). (A) Exposed for PI (DNA). (B) Exposed for fluorescein (TUNEL). (C) Exposed for both PI and fluorescein. (D) Another group of fibers exposed for both PI and fluorescein. Note the twisting of the mature fibers.

Negative control for the TUNEL assays consisted of incubating fiber in TUNEL labeling solution without TdT enzyme solution. Negative control fibers were counterstained with PI and show no fluorescein signal (Figure 15A). For the positive control, DNA strand breaks were induced by incubating fibers with DNase I. This treatment, followed by the same labeling procedure as with uninduced samples, produced TUNEL-positive labeling throughout the fiber length in 10 DPA fibers (Figure 15B). Previous work using DNase I treatment as a positive control has shown positive TUNEL staining in 88% of cells (Groover and Jones, 1999).

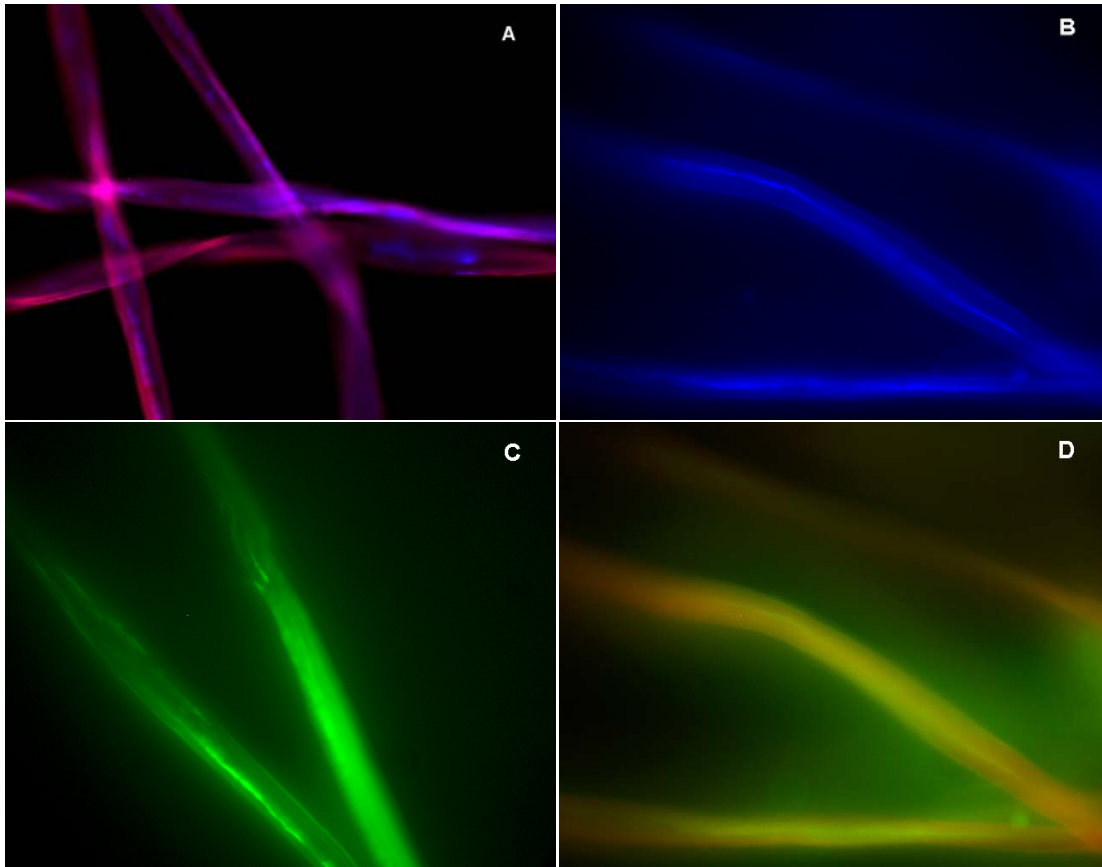


Figure 14. 65 DPA Cotton Fibers.

(A) Exposed under green and UV light for PI (DNA) and fluorescein (TUNEL), respectively. Note the twisting of the dehydrated fibers. **(B)** Exposed under UV light for fluorescein. **(C)** Exposed under blue light for fluorescein. **(D)** Same section as in **(B)**. Exposed under green and blue light for PI and fluorescein, respectively.

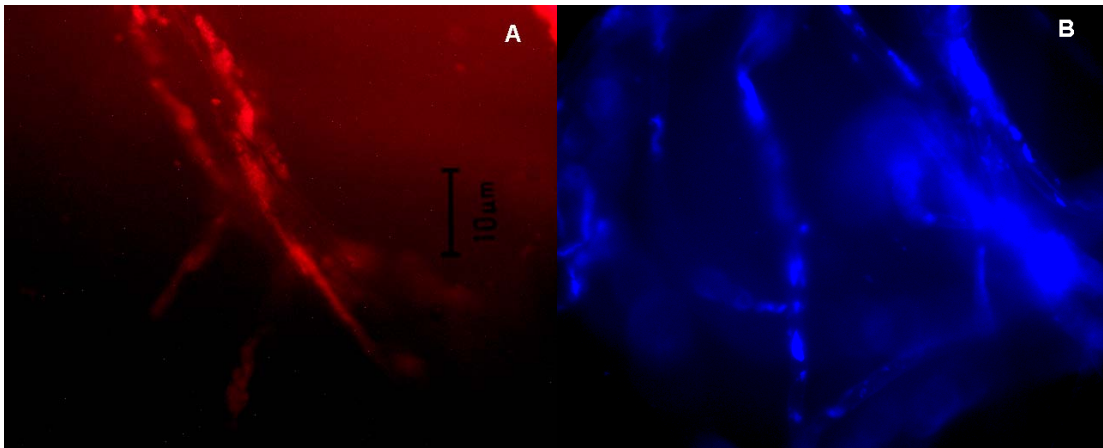


Figure 15. 10 DPA Negative and Positive Control Fibers.

(A) Negative controls were exposed for PI (DNA) and fluorescein (TUNEL). Stained with PI and Label Solution from In Situ Cell Death Detection Kit (Roche) without enzyme solution (TdT). (B) Positive control exposed for fluorescein after incubation with DNase I to induce DNA strand breaks.

As a second positive control, cultured plants of *A. thaliana* were allowed to senesce. Cell death was initiated in this tissue by allowing leaves to grow on MS basal media for a period of 95 days. Senescent leaves were identified by their pale green/yellow coloring. Leaf tissue from the senescent *A. thaliana* culture was stained directly via the TUNEL assay and counterstained with PI (Figure 16). Marginalization of chromatin is a feature of programmed cell death. The TUNEL-positive signal in senescent *A. thaliana* leaves shows a similar pattern (Figure 16B). A different staining pattern occurred between PI and fluorescein dUTP (Figure 16C and D).

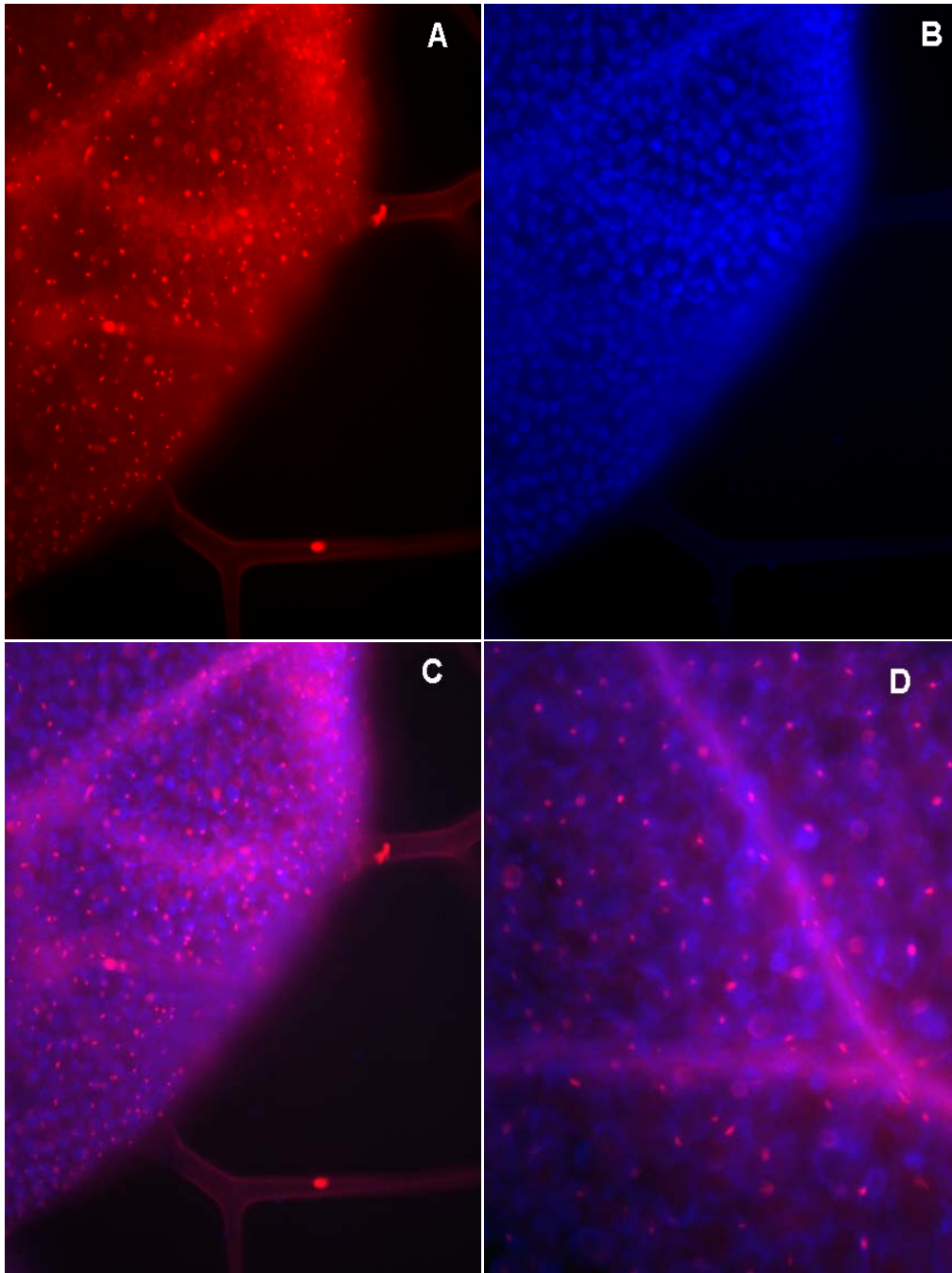


Figure 16. TUNEL Staining of Senescent *A. thaliana* Leaves.

(A) through (C) are the same leaf section. (A) Leaf section exposed for PI (DNA). (B) Exposed for fluorescein (TUNEL). (C) Leaf section showing both PI and fluorescein dUTP staining. (D) Leaf section under higher magnification, with both PI and fluorescein emission.

DISCUSSION

DNA Laddering Assay

There are reported to be approximately 16,000, but upwards of 30,000 fibers per ovule (Taliencio, 2005; Wilkins and Arpat, 2005) and approximately 25 – 35 ovules in a given boll (Beasley and Ting, 1973). In order to visualize a banding pattern on a DNA laddering gel, the Roche Apoptotic DNA ladder kit estimates an ideal cell concentration of 2×10^6 per sample. The requirement for visualization of bands then becomes approximately 4-5 bolls per assay. Given the very large size of cotton fiber cells (2.2-3.0cm x 11-22 μ m) and limited resources, it was unfeasible to incorporate that amount of cells into a single assay.

SYBR Gold staining and increasing the quantity of DNA loaded per gel lane were attempted to enhance the banding image on the gels. After nearly quadrupling DNA volume in gel lanes and staining with SYBR Gold, DNA could only be visualized for 5 – 40 DPA fiber. No bands were apparent in agarose gels after 40 DPA (even with 30 μ L DNA per lane). Even after these additional steps, no laddering could be observed.

Apoptotic DNA ladder gels rely on a substantial portion of cells undergoing DNA fragmentation at the same time (Collins et al., 1997) and reports have shown DNA laddering when no less than 80% of cells were undergoing PCD (Swidzinski et al., 2002). In most experiments, PCD is elicited, allowing for widespread fragmentation across the population of cells. In nature, PCD usually is asynchronous, however. In this experiment, fibers were collected from throughout the boll. As such, the fiber were at varied stages of development. This fact, in conjunction with the difficulty in collecting enough cells to perform a proper assay, could account for why DNA ladders were not apparent.

The possible loss of DNA during the nuclear isolation procedure, compounded with inefficiency of the DNA extraction method are a few possibilities for the low isolation efficiency. It is apparent that the DNA extraction method was inefficient. The

apoptotic DNA ladder kit is meant for quick, as opposed to high volume DNA isolation. In this situation, where cell number required for isolation fell below requirements, it is possible the DNA concentration achieved is so minute, it became nearly impossible to visualize by the means utilized. Low recovery of nucleic acids in maturing fiber cells has been reported due to the thick cell wall (Kim and Triplett, 2001). It may be the case that degradation of DNA in maturing fibers with thickened cell walls is also a reason for low recovery.

Even when a whole genome DNA band was visible, no laddering could be discerned. If the TUNEL-positive signal in microscope images is labeling fragmented DNA, it indicates PCD is not widespread until 45 DPA. This may explain why DNA ladders are not visible for the time points where DNA extraction was successful. As visualization of a banding pattern on an agarose gel requires a high percentage of cells to be undergoing PCD, lack of a laddering pattern does not rule out that PCD is occurring (Collins et al., 1997).

Appearance of Nuclei and DNA

Talercio et al. (2005) stated that fiber nuclei are difficult to observe after the secondary cell wall has formed. At no stage of cotton fiber development may a nucleus be clearly observed. As compared with prior observations of *in vivo* nuclei (Westafer and Brown, 1976), none of the roundness of structure was maintained in stained fibers. Just prior to vacuole rupture in zinnia tracheary elements, however, vacuole expansion pressed the nucleus against the plasma membrane so as to make it appear lengthened and flat (Obara et al., 2001). It is possible that the high turgor during fiber cell elongation produced this flattened nuclear appearance as well. Also, perhaps the pretreatment process, or roughness in handling caused the nucleus to break open causing the DNA to be released into the fiber. As very few observations of late stage fiber exist, it may be that this is the natural state of nuclei in a cotton fiber. It may also be possible that the regions stained by fluorescein dUTP and PI, are not DNA, however, and are actually other cytoplasmic components.

The PI signal between 5 and 20 DPA appears spread throughout the length of the cotton fiber. If this signal may be attributed to DNA, it may be due to the high level of transcription occurring during elongation and secondary wall synthesis. After the onset of secondary wall synthesis, the PI signal condenses, possibly signaling the onset of PCD. In later developmental stages, the TUNEL-positive region was spread throughout the length of the fibers. A similar result was noted in Collins et al., (1997) where cells showed a positive In-Situ Nick Translation (INST) signal that was diffuse, as if the DNA had been released from the chromatin, yet remained in the plasma membrane. Using the same labeling kit applied here, TUNEL-positive elaters of hornworts and liverworts show a highly elongated nucleus (Figure 17) (Kremer and Drinnan, 2003). At maturity, the cotton fiber is 1,000 – 3,000 times longer than it is wide (Kim and Triplett, 2001). The fluorescent regions are likely not as elongated as microscope images, (Figures 3 and 5) would indicate.

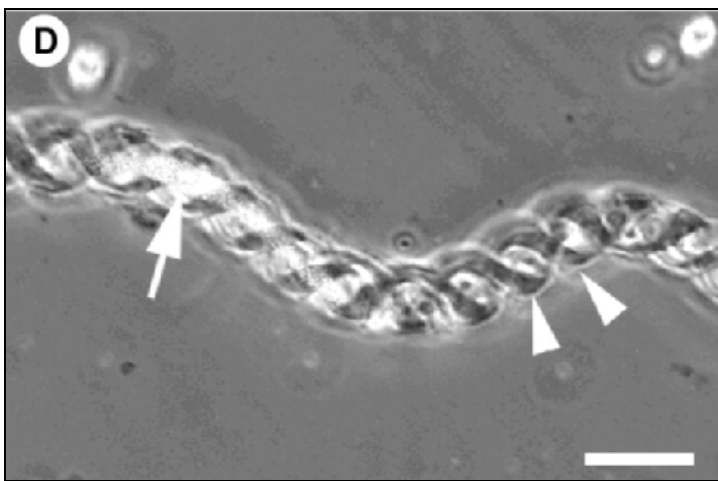


Figure 17. TUNEL Labeling of *Symphyogyna podophylla* (Liverwort) Elator.

Nucleus highlighted by arrow and secondary cell wall highlighted by arrowheads. Note the highly elongated nucleus and the similar shape and structure of TUNEL-positive region in cotton fiber cells. From Kremer and Drinnan, 2003.

Fiber Cell Walls

Fibers in the elongation phase have a thin primary cell wall, noted by faint PI fluorescence (5-15 DPA). While secondary cell walls are being deposited, a TUNEL-positive signal may be observed in the thickening fiber cell walls (20-35 DPA). After completion of secondary cell wall synthesis until maturation, the thickened fiber cell wall fluoresces with PI (40-65 DPA). Further thickening occurs as the fibers dehydrate. Cell wall thickening has been reported as being notable between 30 and 40 DPA, with thickening proceeding until 50 DPA (Waterkeyn, 1981). Mature cotton fibers twist as they dry and the twisting of the fibers can be noted in later DPA fibers, from 50 to 65 DPA. By 65 DPA the cell walls are very thick, with little intracellular space.

In differentiating tracheary elements, TUNEL labeling was only observed in cells with secondary wall thickening and was not observed in pre-differentiated cells. It was even observed that most cells showing secondary wall thickening were not TUNEL labeled, indicating that DNA fragmentation was not initiated until after secondary cell wall synthesis had completed (Groover et al., 1997). Here, fluorescein-dUTP staining only appeared in cells with thickening or thickened secondary cell walls. Contrary to the results of Groover et al., (1997) a study by Wang et al. (1996) showed that developing tracheary elements have a TUNEL-positive signal at the time the elements are initially forming. In 20-25 DPA fibers, a TUNEL-positive signal was detected, however, it appeared to be staining rings of cell wall and not the DNA. Secondary walls of zinnia tracheary elements have been reported to form in annular rings (Ohdaira et al., 2002). It is possible that this signal is not related to fluorescein dUTP, however, and is simply cell wall autofluorescence excited under UV light (Wang et al., 1999). Blue autofluorescence of cell walls may be noted in Figure 20f, a negative control image. Perhaps a similar situation is occurring here, causing fluorescence in secondary wall forming portions. Nuclear fluorescence may be altered due to the cell wall, which is believed to autofluoresce in many cases (Van't Hof, 1999). It cannot be ruled out that intracellular

fluorescence was not quenched somewhat by the fluorescence of the cotton fiber cell wall.

TUNEL and Propidium Iodide Staining

The blue fluorescence in TUNEL assay images as opposed to expected green fluorescence is due to the false image coloring by the MacProbe program. Although fluorescein is typically excited by blue light, it may also be excited by UV light (Schauenstein et al., 1978), both emitting green fluorescence. DAPI is typically excited by UV light, emitting blue fluorescence. As the microscope filter cube used was set for use with DAPI stain, the green fluorescein dUTP fluorescence was false-colored blue in the images. Comparison in fluorescence under blue and UV light excitation show that the same areas are fluorescing in both images (e.g. Figure 10C, D and Figure 18), indicating that this is the likely cause for the blue fluorescence noted throughout the images, and not autofluorescence. Green fluorescence emission images create a sharper image because these pictures were produced under the fluorescein excitation maxima. No other stain was present in the preparations besides fluorescein dUTP and propidium iodide, so blue emission can be attributed only to fluorescein staining.

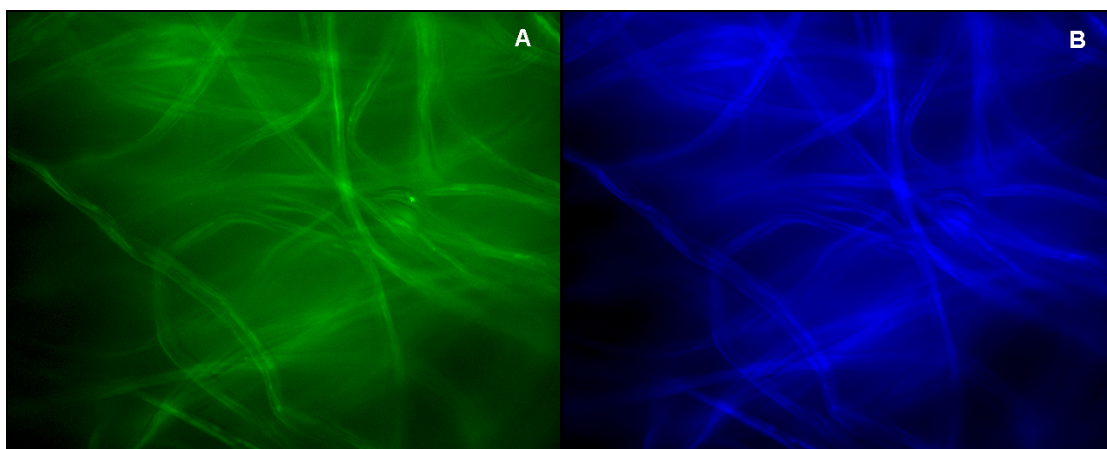


Figure 18. 45 DPA Fibers with Fluorescein Excitation Under Blue and UV Light. **(A)** Fiber under blue light excitation exposed for 0.15 seconds. **(B)** Fiber under UV light excitation exposed for 0.1 seconds. The TUNEL-positive areas are sharper in A, but are in fact, staining the same areas as in B.

Reduced DNA staining is said to occur due to both condensation of chromatin and fragmentation of DNA (Broderick et al., 2002) and PI fluorescence is thought to be reduced by phenolic compounds, such as chlorogenic acid, found present in the cotton fiber (Hendrix and Stewart, 2005). PI staining is consistent until approximately 50 DPA when a great reduction in fluorescence is noted, although the TUNEL signal persisted. As PI will normally only fluoresce red when bound to DNA, reduction in PI signal possibly indicated that the DNA was degrading, although reduction in fluorescence due to interference with phenolic compounds is possible. The total lack of staining in 40 DPA fibers stored at -70° may indicate a complete degradation of DNA and intracellular components, likely due to improper treatment prior to freezing.

Propidium Iodide is a nucleic acid intercalating stain producing red fluorescence when bound to DNA or RNA. Pretreatment with RNase A prevented RNA binding. It is a vital dye, only staining cells without intact plasma membranes, i.e. dead cells. However, as the cotton fibers in this assay were excised from the boll in order to be stained, PI only served as dye to indicate the presence of DNA. In the case of the

senescent *A. thaliana* leaves, the cells were not disturbed in order to complete the TUNEL assay, as such, the PI staining in this case may be considered a dual-stain for apoptosing cells.

Fluorescein and PI had different patterns of staining in senescent arabidopsis leaves. This is possibly due to condensation of DNA, highlighted by PI and the fragmentation of DNA and its distribution to the periphery of the nucleus, highlighted by fluorescein dUTP, although other possibilities remain. A negative control using young arabidopsis leaves would likely have answered some questions regarding the different staining pattern between PI and TUNEL. The pattern of fluorescence in senescent *A. thaliana* leaves could represent a distinct and observable phase of programmed cell death (membrane blebbing). Note, in Figure 19, the significant similarity in fluorescent TdT labeling pattern to senescent arabidopsis leaves (Figure 16) (Gorczyca et al., 1993).

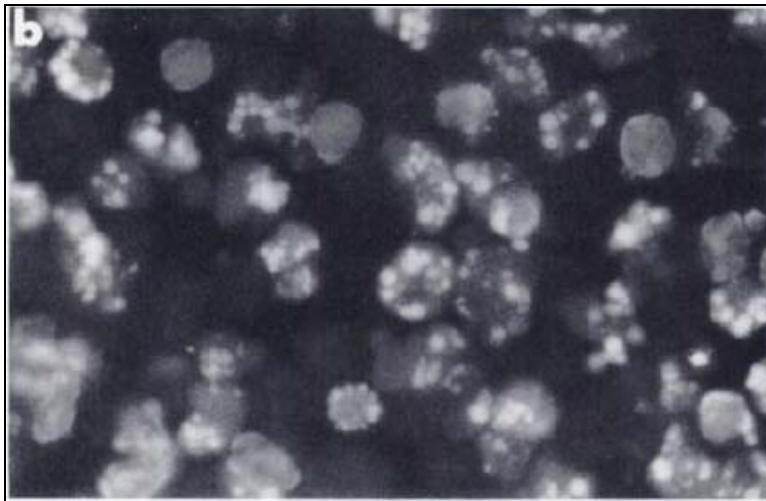


Figure 19. Apoptosis in HL-60 Cells.

HL-60 cells treated with 0.15 μ M DNA topoisomerase inhibitor, camptothecin for 4h and labeled with terminal transferase and b-dUTP, counterstained with fluoresceinated avidin. From Gorczyca et al. 1993.

The fixation and embedding processes used in the TUNEL staining procedure in plants have been reported to cause a false-positive signal due to accidental DNA strand breakage (Wang et al., 1996; Collins et al., 1997). It is possible that, although there was no embedding in this experiment, that some false-positive signal was produced, as seen in TUNEL-positive staining in young fibers (Figure 20C). In most all samples, where a fiber segment or debris was present, it was intensely stained with fluorescein-dUTP. This may be due to contamination of TUNEL-positive fibers into TUNEL-negative samples (Figure 20D and E). Debris was also present in control samples (Figure 20A). In the TUNEL-negative samples that did show errant TUNEL-positive fibers, the fibers appeared damaged in comparison to the general fiber appearance (Figure 20B, G, H). Although it is likely that contamination with TdT caused the TUNEL-positive labeling in Figure 20I, it is possible that the blue signal in Figure 20F is due to autofluorescence of the cell wall (Wang et al., 1999).

The first attempt at TUNEL labeling in cotton fibers, was met both success and failure. The procedure produced bright, positive TUNEL staining in different stages of development, however, as fluorescent images were not accompanied by phase contrast or light microscopy, it was impossible to tell whether or not the stained areas were actually fragmented DNA. Secondary cell wall formation and drying and twisting of mature fibers was visible despite nonspecific labeling of intracellular space, allowing for unique and interesting observations to be made; observations that will likely become unambiguous with further work.

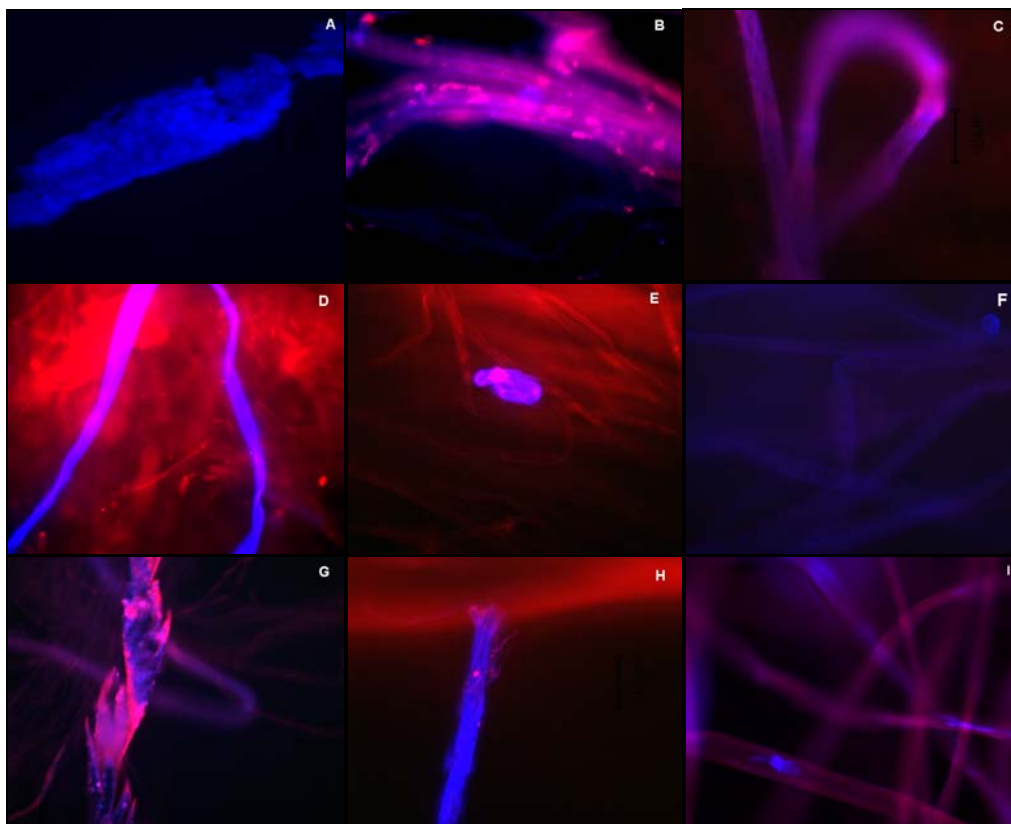


Figure 20. Anomalies in TUNEL Staining of Cotton Fibers

All images exposed for PI and fluorescein, unless otherwise noted. **(A)** TUNEL-positive debris from 15 DPA positive control sample. Exposed for fluorescein. **(B)** 10 DPA fiber. Note the jagged appearance to the fiber, indicating it had become damaged at some point. **(C)** Errant TUNEL-positive fiber in 10 DPA sample. **(D)** Errant TUNEL-positive fiber in 15 DPA sample amid TUNEL-negative fibers. **(E)** Errant TUNEL-positive fiber in 20 DPA sample. **(F)** TUNEL-positive cell walls of 20 DPA negative control. Exposed for fluorescein. **(G)** Highly TUNEL-positive fragment in 25 DPA fiber sample. This item has obviously undergone mechanical damage. **(H)** Highly TUNEL-positive fiber in 30 DPA sample. Fiber has likely undergone damage, note the frayed end. **(I)** 45 DPA negative control sample with TUNEL-positive areas. Likely due to contamination during the staining process.

CONCLUSION

The staining patterns produced by both PI and TUNEL stains do not allow conclusive deductions to be made. TUNEL-positive and PI-stained areas were observed, but failure at any time-point to observe conventionally shaped nuclei in my cytological preparations, along with possible nonspecific staining or autofluorescence of cell wall and intracellular components, made it difficult to draw firm conclusions as to their significance. As such, additional analyses will be needed to prove or disprove current PCD theories.

Differences in TUNEL and PI signals across fiber development stages indicate that the observed fluorescence patterns are marking discrete developmental phases, however. The PI signal is dispersed throughout the cell during the elongation phase (5-15 DPA) and appears to condense during secondary cell wall synthesis (25-40 DPA). Secondary wall synthesis is notable by 20 DPA. An intracellular TUNEL signal does not occur in a large percentage of fibers until 45 DPA. At 50 DPA and beyond, PI staining is reduced.

Fiber DNA extraction may be accelerated by pre-extraction of nuclei, although the method used here was low in efficiency. Perhaps, with a higher efficiency subsequent DNA extraction method, DNA may be isolated throughout development in higher quantities and past the 40 DPA time point, possibly allowing for visualization of a DNA ladder.

As in tracheary elements, secondary wall formation is most likely temporally related to PCD, as indicated by the coordinated emergence of AGPs, Ca^{2+} , hydrogen peroxide and brassinosteroid. The results, although inconclusive, point to the possibility that PCD may be a process leading to maturation in the cotton fiber, succeeding completion of secondary cell wall synthesis.

METHODS

Plant Material

20 *Gossypium hirsutum* TM-1 plants were grown in pots under greenhouse conditions. Flowers were tagged on the day of anthesis. At specific days post anthesis, bolls were collected in 5-day increments for experimentation beginning at 5 DPA and continuing through 65 DPA. Several time-points were collected at each staining date (7/28/06, 8/2/06, 8/25/06, 9/3/06, 9/26/06) and each time-point was sampled at least twice. Immediately after collection, whole fibers from throughout the boll were carefully pulled from the ovules so as to avoid contamination with ovule cells and used for subsequent processing.

Arabidopsis thaliana var. Columbia (wild type) were grown on Murashige and Skoog (1962) basal salt media (MS). Cultures maintained in MS media for a period of 95 days were used to assay senescent leaf tissue. Tissue samples were used directly for TUNEL staining.

Nuclear Isolation

The procedure for nuclei extraction, provided by Taliercio et al., (2005), a modification of the protocol by Price and Johnston, (1996) was modified further for this experiment. Fibers were carefully removed in order to avoid contamination with ovule tissue (except in the samples where ovule tissue was desired). For nuclei extraction, approximately 0.5 g of fibers were placed into a petri dish and 2 ml cold (4°C) nuclear isolation buffer was added (45 mM MgCl₂, 20mM MOPS, 30 mM sodium citrate, 0.1% Triton X-100 and 2.0% PVP-40, pH 7.0). Just prior to each isolation one Roche complete protease inhibitor tablet was added per 10 mL nuclear isolation buffer. Tissue was chopped with a razor blade for approximately 2 min. Plates were then placed on a rotary shaker for 5 minutes to allow cell debris and nuclei to dissociate, after which the homogenate was

poured into a 50ml falcon tube covered with Miracloth. The petri dish was rinsed with 1 mL nuclear isolation buffer and the Miracloth was squeezed to retrieve buffer that had not yet drained. The filtrate was then re-filtered through a 50 mL syringe fitted with 17 μ m nylon filter into a 1.5 mL snap-cap tube. The samples were then centrifuged in a standard centrifuge at 4°C for 5 min at 1000 rpm. The supernatant was discarded and the pellet was re-suspended in 200 μ L isolation buffer for DNA extraction. 3 μ L RNase A (1 mg/mL) was added and the samples were kept at -20°C until further processing.

DNA Extraction and Gel Electrophoresis

For DNA extraction and visualization the Roche Apoptotic DNA ladder kit was used with isolated nuclei. The samples, along with positive control apoptotic U937 cells, were first lysed in binding buffer (6M guanidine-HCl, 10mM urea, 10mM Tris-HCl, 20% Triton X-100 (v/v), pH 4.4). After incubating for 10 minutes, 100 μ L isopropanol was added and the sample was vortexed, then transferred to microcentrifuge tubes (from the kit) containing glass fiber fleece for binding nucleic acids. The samples were centrifuged and rinsed several times with washing buffer (20mM NaCl, 2mM Tris-HCl, pH 7.5, in ethanol). The samples were eluted with warmed (70°C) elution buffer (10mM Tris, pH 8.5). This step released the DNA from the glass fiber fleece, freeing DNA for analysis. Sample DNA along with a positive control of lypophilized apoptotic U937 cells were run on 2% agarose in TBE gel with EtBr and was run in a gel chamber with TBE buffer (0.089 M TRIS, 0.002 M EDTA, 0.089 M Boric Acid, pH 8.3). After running (~1.5 H @ 75V), gels were further stained with SYBR Gold (5 μ L in 50 mL TBE) for 1hour in the dark. Gels were visualized with a UV light box.

TUNEL Assay

Negative control samples were produced from various developmental stages (10, 15, 20, 30, 40, and 45 DPA). Positive control samples were also taken from several

developmental stages (10, 20 and 40 DPA). As a second positive control, senescent *A. thaliana* leaves were stained with the TUNEL reaction mixture.

For staining of apoptotic fibers, the In Situ Cell Death Detection Kit, fluorescein (Roche) was used. Some modifications were made to the outlined protocol. NRB buffer (50 mM Tris-HCl (pH 7.8), 5mM MgCl₂, 10 mM β -mercaptoethanol and 20% glycerol) (Luthe and Quatrano, 1980) was substituted for PBS and 1.5 mL snap-cap tubes were used in place of 96-well plates due to the large cell size. Fixation solution was prepared with 4% paraformaldehyde in NRB, pH 7.4. Permeabilisation solution contained 0.1% Triton X-100 in 0.1% sodium citrate. Fixation and Permeabilisation solutions were prepared fresh prior to each staining procedure. Cotton fiber tissue (ovule or leaf tissue in some cases) was collected and placed in a 1.5 mL tube, then washed 3 times in NRB. After which, fixation solution was added to each sample and they were allowed to incubate for 1h on a shaker. Samples were then centrifuged at 2,000 RPM for 10 min and fixation solution was removed. Cells were washed once with NRB and permeabilized for 2 min on ice with permeabilisation solution. Cells were washed twice more with NRB and re-suspended in TUNEL reaction solution (except negative controls) in a dark, humid atmosphere for 1 hour at 37° C. TUNEL reaction solution (reagents included in kit) contained 1 part Enzyme solution to 9 parts Labeling Solution. Negative-control samples were incubated with Label Solution, instead of TUNEL reaction mixture. Positive-control samples were incubated with DNase I, grade I for 10 min prior to labeling, to induce DNA strand breaks. Samples were washed twice more with NRB and placed in a final volume of 300 μ l NRB. 30 μ L 1mg/ml propidium iodide was then added. Unless otherwise noted, all samples were stained with both PI and fluorescein dUTP.

Samples were viewed on an Olympus AX70 fluorescence microscope. PI fluorescence was excited by light at approximately 536 nm (green) and viewed at a wavelength of approximately 617 nm (red). Fluorescein dUTP was viewed at wavelengths of approximately 518 nm (green) and 461 nm (blue), with excitations wavelengths of approximately 494 nm (blue), and 358 nm (UV), respectively. Images

were captured with a Sensys digital camera mounted to the microscope. Images were viewed and analyzed using MacProbe 4.2 and exported as TIFF image files.

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